

VISUALIZATION OF STIMULATED NERVE ENDINGS BY PREFERENTIAL CALCIUM ACCUMULATION OF MITOCHONDRIA

A. PARDUCZ and F. JOÓ. From the Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, H-6701 Szeged, Hungary. Dr. Parducz's present address is the Department of Neurosciences, McMaster University, Health Sciences Centre, Hamilton, Ontario, L8S 4J9, Canada.

An understanding of the regulation of depolarization secretion coupling depends in part on the recognition of the intracellular localization of calcium-binding sites. Although calcium ions are known to be essential for releasing neurotransmitters, it has not so far been revealed if, in certain circumstances, mitochondria of stimulated nerve terminals accumulate calcium preferentially in the form of electron-dense granules.

In a research project aiming toward the elucidation of the changes occurring in synaptic ultrastructure in relation to neurohumoral transmission (3, 15, 16), we have studied the effect of fixatives on the preservation of synapses in the superior cervical ganglion (SCG) of the cat. Yates and Yates (22) first showed that adding calcium to the

fixative would preserve electron-dense granules in nerve mitochondria. The finding was confirmed by Sampson et al. (19) and became well established in the Oschman and Wall technique (14) for revealing the sites of calcium deposition in tissues. We report here that with the use of a calcium-containing fixative (21) it is possible to distinguish between electrically stimulated and nonstimulated nerve endings, on the basis of presynaptic calcium accumulation of mitochondria.

MATERIALS AND METHODS

Cats were anaesthetized with 40 mg/kg sodium pentobarbital. The cervical sympathetic trunks were exposed and stimulated by square impulses of the following parameters: frequency 20/s, duration 1 ms, amplitude

6–8 V. Contractions of the nictitating membrane were recorded on a kymograph. Stimulation was carried out for 1, 5, and 20 min. Small cubes of stimulated and control ganglia were prefixed in a 10% paraformaldehyde fixative buffered with *s*-collidine containing 0.005 M calcium chloride (21) at 4°C for 2 h, then followed by postfixation in calcium-free 2% osmium tetroxide buffered with 0.2 M *s*-collidine. In some cases, fixation of stimulated samples was carried out from the beginning at 56°C. Samples were then dehydrated in calcium-free graded ethanols and embedded in Durcupan (Fluka AG, Basel, Switzerland). Thick sections up to 0.5 μm were used for energy dispersive X-ray analysis. Sections were coated with carbon and viewed in a JEOL 100B transmission electron microscope equipped with a scanning attachment (JEM-ASID) and an EDAX energy dispersive spectrometer. This analytical system allowed viewing of the image and localization of the beam for excitation of a spot approximately 200 Å in diameter. To express the extent of granule formation in the mitochondria of stimulated nerve terminals, the percentage of area occupied by granules was determined. It should be noted that no significant changes in mitochondrial volume (swelling, shrinkage) were observed after stimulation.

RESULTS AND DISCUSSION

The characteristic appearance of the electron-dense granules in the mitochondria of a stimulated preganglionic nerve terminal are shown in Fig. 1. The dense granules were confined to the matrix around the cristae and other regions of the inner membrane. Similar granules were not seen either in the mitochondria of the postsynaptic dendrites or in those of Schwann cells. Occasionally, electron-dense granules were observed in the mitochondria of perikarya of some ganglion cells, particularly when the stimulation was carried out for 5 min. In control ganglia (Fig. 2), the mitochondria of the presynaptic endings sometimes contain granules but these are of smaller size and less electron density. The comparison of X-ray spectrometer signals, obtained from mitochondria of different processes within a given section, clearly demonstrated that mitochondria with electron-dense granules were significantly higher in calcium (Fig. 3*a*) than any other mitochondria located in different elements (Fig. 3*b*). From Table I, it is clearly seen that longer stimulation resulted in a larger area occupied by granules in the presynaptic mitochondria. However, significantly fewer and smaller dense granules were observed in presynaptic mitochondria when fixation of the stimulated samples was carried out at 56°C from the beginning.

It has been suggested by Lehninger (9) that mitochondria are capable of accumulating massive

amounts of calcium. It was evidenced by Peachey (17) and Greenawalt et al. (4) that calcium uptake leads to the appearance of electron-dense granules in fixed mitochondria. This phenomenon supports Weiss' original idea (20) that these granules are cation exchange resins or binding sites. As to the mechanism of the development of calcium, it is presumed that high-calcium fixatives may reduce the extraction of calcium from these sites during fixation, since electron-dense granules are present in mitochondria of totally unfixed, frozen tissues (2). It is also conceivable, however, that the fixative could deposit unnatural amounts of calcium at newly exposed anionic binding sites. Neuronal mitochondria have been shown (11) to take up calcium and develop dense granules when exposed to high calcium solutions (5). The calcium accumulation described in this paper was exclusively presynaptic, which is consistent with Katz' evidence (8) that calcium enters the nerve terminal to trigger transmitter secretion during nerve stimulation. In addition, this presynaptic calcium accumulation is in good agreement with Blaustein's (1) and Llinas' (12) direct evidence that calcium enters the presynapse during stimulation or depolarization, or could be a natural consequence of this calcium entry. Preferential calcium accumulation in mitochondria represents one way of distinguishing stimulated nerve terminals in addition to depletion of synaptic vesicles and expansion of the presynaptic membrane resulting from exocytosis of synaptic vesicles, which changes were observed earlier (6, 18).

In view of later results (7, 13), it is conceivable that calcium-containing electron-dense structures, revealed by using the Oschman and Wall technique (14), are closely related to the calcium-binding protein. In this sense, calcium accumulation in the mitochondria of stimulated synapses of the SCG, visualized in our case by using another aldehyde fixative, may represent the activation of calcium-binding protein due to the enhanced influx of external calcium during depolarization. The stimulation dependence of this phenomenon is further supported by the observation that the number of granules does not change but that they become larger with the duration of stimulation. The fact that calcium accumulation in mitochondria was inhibited by heat treatment may indicate that stimulation produced a change in state which caused the mitochondria to accumulate calcium from the fixative. It is well known, from the work of Lehninger et al. (10), that different divalent cations can accumulate in mitochondria and that

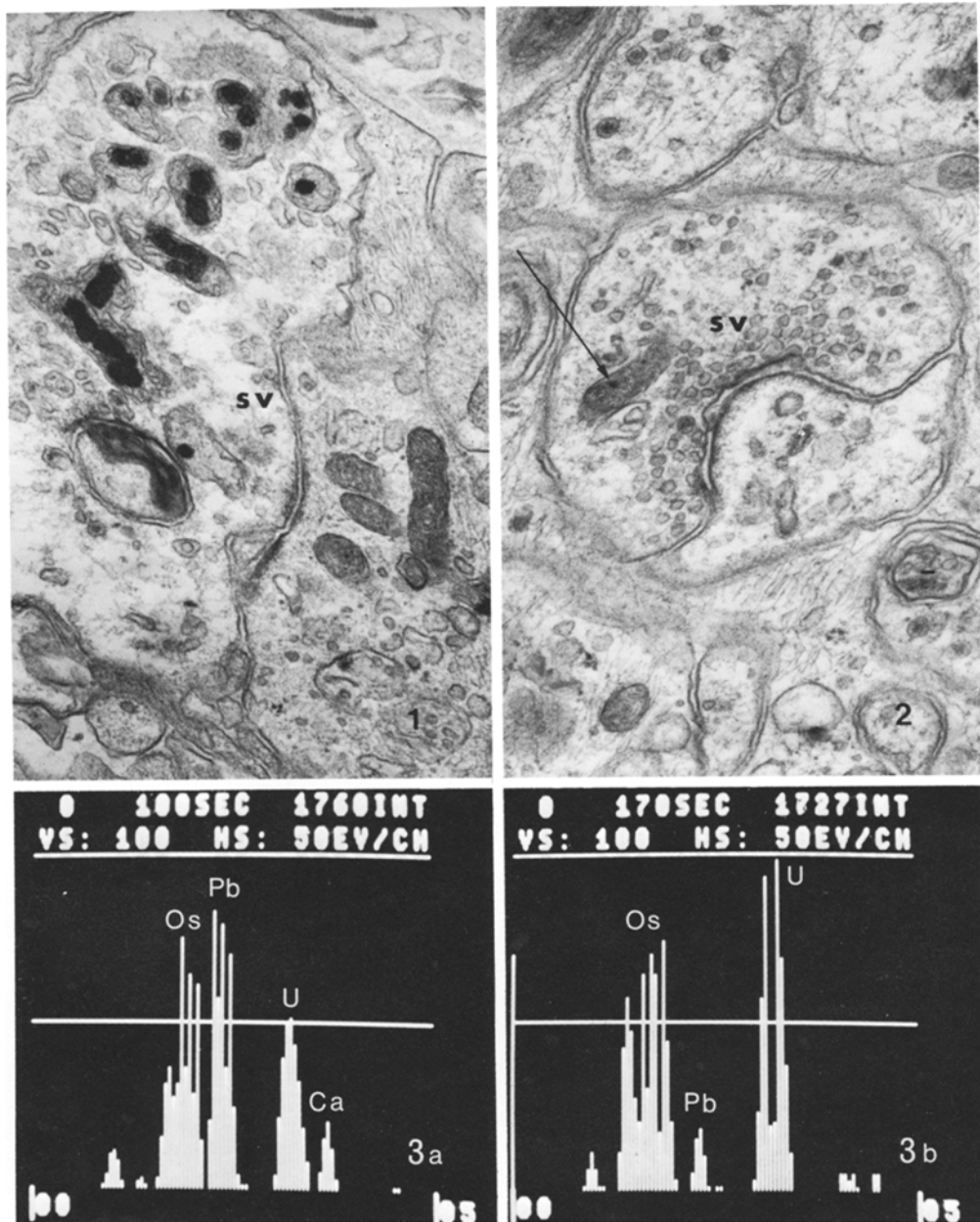


FIGURE 1 A synapse from the superior cervical ganglion of cat stimulated electrically with 20/s for 20 min and prefixed in 10% paraformaldehyde containing 0.005 M calcium chloride. Several large, electron-dense granules are seen in the presynaptic mitochondria. Synaptic vesicles (sv). $\times 34,000$.

FIGURE 2 Ganglionic synapse from a control ganglion. Arrow points to a granule in the presynaptic mitochondrion. Synaptic vesicles (sv). $\times 34,000$.

FIGURE 3 Energy dispersive X-ray spectrographs of the same synapse as seen in Fig. 1, but obtained from another section. Fig. 3 a was taken of an electron-dense granule confined to a mitochondrion of a presynaptic terminal, while Fig. 3 b was obtained from the mitochondrion of a postsynaptic dendrite. After the analysis was completed, the background was subtracted from the spectra by the EPIC module. Calcium was detected in the mitochondrial granule of the stimulated nerve ending only. Os comes from the fixative, U and Pb from the staining.

TABLE I
Calcium Accumulation in the Mitochondria of Nerve Endings

Groups analyzed	No. of synapses evaluated	No. of mitochondria found in synapses	Actual no. of granules	Area occupied by granules
				% ± SD
Control	26	50	44	2.13 ± 1.33
1 min	23	50	104	3.80 ± 2.71
5 min	27	50	116	6.93 ± 4.85
20 min	29	50	88	12.81 ± 7.56

the uptake of ions leading to granule formation is energy dependent. Whether or not the preferential calcium accumulation observed in our study is connected with the production of energy by mitochondria is yet to be determined. It can be seen, even at this early stage of investigation, that preferential accumulation of calcium reflects indirectly an ultimate difference between the pre- and postsynaptic membranes with respect to their calcium uptake.

SUMMARY

Calcium was detected by X-ray microanalysis in the mitochondria of electrically stimulated nerve endings. The phenomenon described here offers a simple means for identifying the stimulated nerve endings in the electron microscope and appears to be a promising new method for following spontaneous and drug-stimulated translocation of calcium in relation to the regulation of neurotransmitter release.

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